CHAPTER 2 - LITERATURE REVIEW

2.1 INTRODUCTION

Enzyme-modified cheese (EMC) is a powder or paste derived from cheese or curd using enzymes, and is used as a cheese replacer in certain foods such as processed cheese, cheese sauces, spaghetti sauces, soups and dips (Sutherland, 1991). EMC may replace 20 to 50 times their weight of natural cheese in a food product and are cheaper to produce than cheese (Fox, 1998). Hence their primary reason for production is economy, since cheese is an expensive food ingredient (Main, 1991). EMC provides the food manufacturer with a product that is cost effective, has a natural flavour and is also nutritious as compared to non-nutritive pure chemical-based flavours (Moskowitz & Noelck, 1987). In the United States, EMC has GRAS status (generally regarded as safe) and as such can be added to a variety of foods (Freund, 1995).

From Table 1 it can be seen how Cheddar EMC fits into the variety of Cheddar cheese products available and that EMC is valued for its flavour rather than its texture and flavour as in other cheese products. EMC is a form of accelerated ripened cheese, ripened to the point where the enzymatic breakdown becomes excessive and texture quality is lost (Fox, 1998). Due to this loss of texture quality EMC is produced either as a paste or a spray-dried powder (Moskowitz & Noelck, 1987). Manufacturing the EMC as a paste also increases the available area for enzymolysis.

Henceforth, unless mentioned to the contrary, any reference to cheese or EMC should be taken as referring to Cheddar cheese and Cheddar EMC respectively.

2.2 CHEDDAR CHEESE, IT'S HISTORY AND RELEVANCE TO THE FOOD INDUSTRY

Cheese has been known since antiquity, is mentioned in The Bible (2 Samuel 17:29, Job 10:10) and is believed to have originated in the "Fertile Crescent" between the Tigris and Euphrates rivers in Iraq, some 8000 years ago. There are over 500 varieties of cheeses available world-wide with new varieties being discovered/created every year (Fox *et al.*, 1996). No other cheese is as well known as Cheddar cheese, which originated in the town of Cheddar in Somerset, England. In Elizabethan

Table 1 Comparison of different types of Cheddar cheese products

Product	Process used	Importance
Cheese	Traditional cheese production.	Good as a savoury flavour, used "as is" or in a variety of products (Sutherland, 1991).
Accelerated ripened cheese	Traditional cheese production with added enzymes or elevated maturation temperatures (Fox *et al.*, 1996).	Reduced maturation time, which reduces inventory costs (Kilara, 1985). Must taste the same as traditional cheese.
Processed cheese	Cheese processed by addition of emulsifiers to make a smooth "plastic" paste.	Good shelf life and easily incorporated into food products (Mann, 1993).
Cheesebase	Highly accelerated production of liquid cheese products from ultrafiltration concentrated milk in 12-24 hours, then pasteurised.	Simulates flavour of a young cheese. Can be spray dried but low flavour strength (Sutherland, 1991).
Enzyme-Modified Cheese	Curd slurry technique (Kristoffersen *et al.*, 1967; Singh & Kristoffersen, 1971a).	High strength, low-cost product simulates mature cheese flavour with mouthfeel and texture not important. Taste, aroma and flavour important (Moskowitz & Noelck, 1987; West & Pawlett, 1996).

times, Thomas Fuller described Cheddar cheese as "the best and biggest in England" with his one regret being "they were so few and so dear, hardly to be met with save at some great man's table" (Anon, 1991). Cheddar cheese is in high demand for its good savoury flavour (Sutherland, 1991).

Of the estimated 480 million tonnes of milk produced world-wide in 1988, 33% went into cheese resulting in 13.25 million tonnes of cheese with 25% of the cheese being Cheddar or its different varieties (Anon, 1991). In 1991, South Africa's cheese consumption was estimated at 1.8 kg per person per annum, which is far less than the largest consumer, France, where consumption is 22.8 kg per person per annum (International Dairy Federation, 1993).

The Australian Dairy Corporation in 1990 (according to Sutherland, 1991) stated that of the 126 600 tonnes of cheese produced in Australia, 49% was sold "as is" to consumers, 21% was used for making processed cheese and 30% was used in industry and food service sectors for further processing. Hard cheese is used in products like pizzas, cheese sauces, bread topping, sausages, pasta dishes and microwave meals while soft cheese is used in cheesecakes, flans and chilled desserts. This use of cheese in the food processing industry has also resulted in the formulation of

new dairy products e.g. cheese flavour (from enzyme-modified cheese), processed cheese and cream flavour (enzyme-modified butterfat) (Main, 1991).

Cheese has special legislation over and above the standard laws applying to the production of food, which cover amongst other factors, the starter bacteria and enzymes permissible in cheesemaking. The International Dairy Foundation (1990) published a report that summarises the legislation covering enzyme and starter use in many countries. As of the report date, South Africa had no regulations controlling the use of enzymes or starters in cheesemaking.

2.3 CHEDDAR CHEESE FLAVOUR FORMATION

The flavour of cheese has been studied since the beginning of the 20th century but it wasn't until gas chromatography (GC) was invented in the 1950s that the many different volatile chemicals in cheese could be detected (Fox *et al.*, 1996). Once the volatile and non-volatile chemicals that were responsible for flavour and taste could be detected, it needed to be ascertained which of the many chemicals were important for cheese flavour. From this information the key chemicals for some cheese flavours were discovered. Moskowitz & La Belle (1981) stated that the key flavour chemicals for Roquefort cheese are 2-heptanone, 2-nonanone and 2-undecanone. This easy characterisation of blue cheese flavour compounds in general is due to the fact that the main flavour compounds come from the specific fungi used in their production (Vafiadis, 1996). Cheddar cheese on the other hand does not have any characteristic chemical flavour but rather has a flavour profile or fingerprint and Mulder (1952) (according to Aston & Dulley, 1982) called this phenomenon the component balance theory.

Much work has been done on Cheddar cheese flavour with some authors observing that certain chemicals or classes of chemicals such as FFA were of key importance to cheese flavour since they increased with cheese flavour (Singh & Kristoffersen, 1970). Other authors found little or no relationship between FFA and flavour (Reiter, Fryer, Sharpe & Lawrence, 1967) implying that the relationship between FFA and flavour is more complex than a simple proportional one. Fox *et al.* (1996) report that many recent authors have concluded that "the flavour of cheese depends on a weighted concentration ratio of all components present". This statement is similar to the component

balance theory proposed by Mulder 40 years ago, and so we are no closer to finding what the key chemicals are (if any) that cause Cheddar cheese flavour (Delahunty, Crowe & Morrisey, 1996).

Ney (1981), Fox (1989) and Wilkinson (1993) stated that cheese flavour comes from the glycolysis, lipolysis and proteolysis of the carbohydrates, lipids and proteins and in milk with the importance of each process varying between the different varieties of cheeses. Flavour compounds are also formed from inter-reactions between the substrates and products of glycolysis, lipolysis and proteolysis (Sharpe, 1979). Proteolysis is perhaps the most important biochemical event that contributes to Cheddar cheese flavour in contrast to some of the blue cheeses where lipolysis is the key event (Fox & Wallace, 1997). A summary of the main events that take place in Cheddar flavour formation is shown in Figure 1. Each of the different processes will now be discussed individually.

2.3.1 Glycolysis

The glycolytic breakdown of lactose in milk to lactic acid is important for the making of cheese and this is done by starter lactic acid bacteria (LAB) and non-starter LAB (NSLAB) (Fox *et al.*, 1996). The low pH created by the lactic acid, as well as the action of the rennet (typically chymosin) on the caseins, leads to the formation of curd due to the breaking of the colloidal suspension (see the section on proteolysis for more details). Cows milk typically has 5% lactose (Ney, 1981) and during Cheddar curd formation 98% of the lactose (either as lactose or lactic acid) is lost during whey draining resulting in a lactose/lactic acid level in the cheese of 1-1.5% (Fox & Wallace, 1997).

Lactose and lactic acid are the only active flavour compounds in fresh cheese curd since the casein and fats are bland (Ney, 1981). In the glycolytic pathway of lactose metabolism the intermediate compound is pyruvic acid (Ney, 1981) which also gets converted by the LAB and NSLAB to acetic acid, diacetyl, acetaldehyde, ethanol and CO_2 (Ney, 1981; Law, 1984). Lactose is also broken down into its constituent products of galactose and glucose but the levels of these sugars are below the flavour threshold (Ney, 1981). Lactic acid has taste in its own right (Fox, McSweeney & Singh, 1995a), and Kielwiein & Daum (1979) (according to Ney, 1981) stated that there must be more L(+)-lactic acid than D(-)-lactic acid for taste. Fox *et al.* (1995a) disagree with Kielwiein & Daum and stated that the racemization of lactic acid is unimportant from a taste point of view.

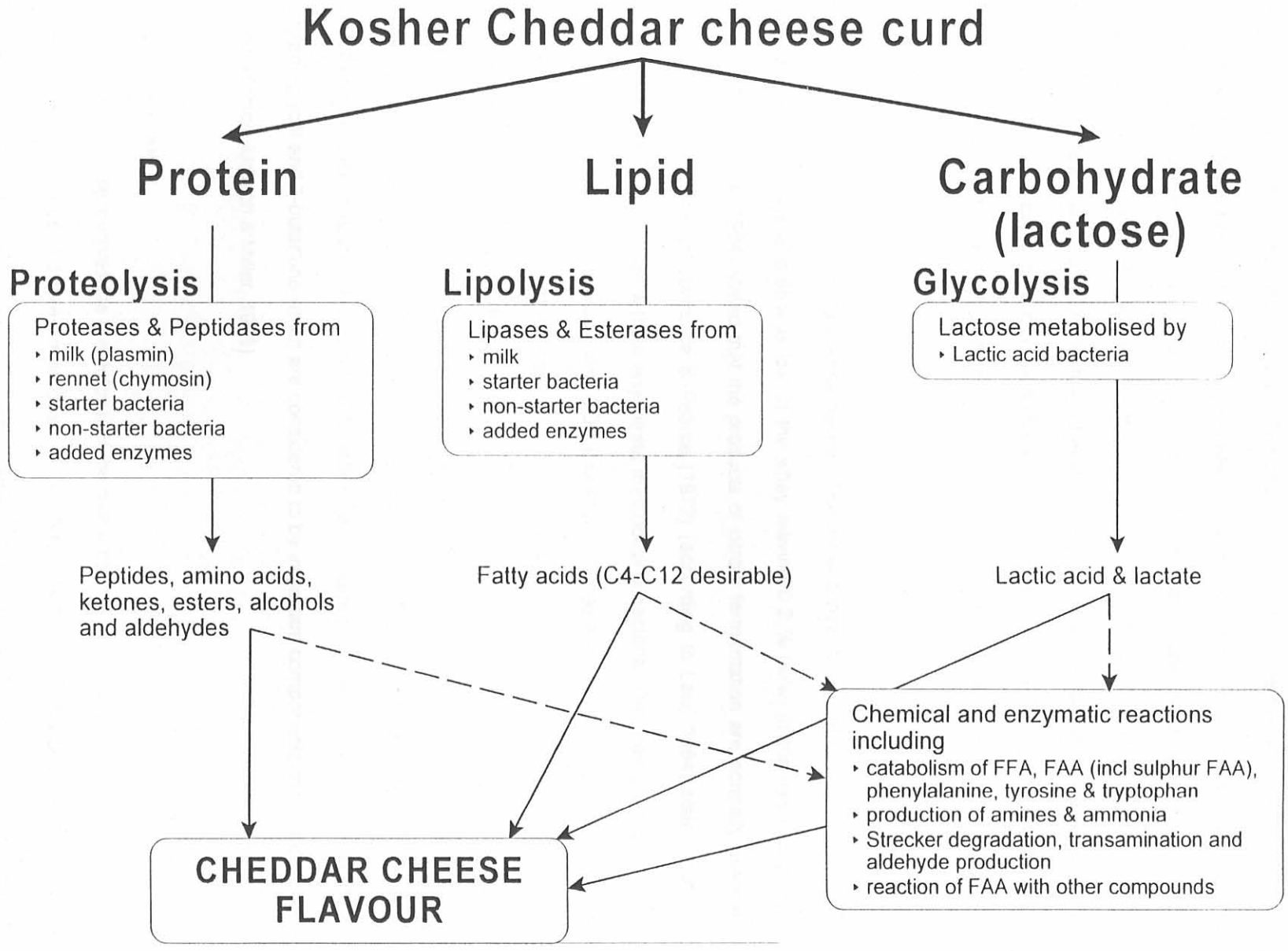

Figure 1 Formation of flavour compounds in Cheddar cheese (adapted from Fox *et al.*, 1996; Fox & Wallace, 1997)

A very important effect of the lactic acid production is the lowering of the pH with Fox *et al.* (1995a) stating that this is the key parameter for good flavour development in cheese since it has follow-on effects. The pH has an effect on essential and non-essential microorganism growth, enzyme function as well as the various biochemical reactions that occur during cheese ripening (Fox & Wallace, 1997). Artificially increased lactose levels during cheese production lead to faster flavour development though the resultant flavour is harsh (Fox & Wallace, 1997). Fox *et al.* (1993) stated that good cheese flavour is a result of rapid and complete metabolism of any residual lactose in the cheese.

2.3.2 Citrate metabolism

Citrate metabolism is important to cheese flavour. The initial concentration of citrate in cows milk is approximately 1.8 g/litre and 95% is lost in the whey leaving 0.2 % (w/w) in the fresh curd (Fox & Wallace, 1997). Law (1984) stated that the products of citrate fermentation are normally associated with cheese aroma though Lawrence & Pearce (1972) (according to Law, 1984) stated that good Cheddar cheese can be made without any citrate metabolising bacteria. The main products of citrate fermentation are diacetyl, acetic acid and CO_2 (Fox *et al.*, 1995a) and formate (Fryer, 1970). Excess CO_2 leads to bad quality Cheddar cheese (called blowing) but a limited amount is required in Dutch-type cheese for eye production (Fox & Wallace, 1997). Acetic acid is important for Cheddar cheese flavour though excess will cause off-flavours (Ney, 1981; Aston & Dulley, 1982). Diacetyl gives a creamy flavour, is essential in butter and yoghurt (Moskowitz & La Belle, 1981) and is important for good Cheddar cheese flavour (Moskowitz & Noelck, 1987). Diacetyl is also converted to acetoin, 2,3-butylene glycol and 2-butanone which are considered to be important components of Cheddar cheese flavour (Dimos, Urbach & Miller, 1996).

2.3.3 Proteolysis

Proteolysis in cheese involves the breakdown of the milk proteins (mostly casein) sequentially to large peptides, small peptides and free amino acids (FAA) (Ney, 1981; Fox & Wallace, 1997). The other non-casein proteins that occur in milk (α-lactalbumin, β-lactoglobulin, serum albumin, immunoglobulins and proteose-peptone components) are left in the whey during curd formation and as such are called whey proteins. Whey proteins are only involved in cheese production when the cheese is made from ultra-filtered milk, where the milk is concentrated before the production of the

curd. For this review, only cheese that has not been made from ultra-filtered milk will be considered for discussion so the contribution of whey proteins to flavour will not be discussed. Proteolysis is the primary and most complex of the biochemical reactions contributing to Cheddar cheese flavour (Fox *et al.*, 1996).

Casein is a phospho-protein complex composed of α, β, κ and γ-caseins associated in micelles (Fox, 1981) and Nagodawithana (1995) stated that κ-casein is well known for its ability to stabilise the micelles in suspension. Rennet is added to cheese together with the LAB to form curds. Rennet consists mostly of chymosin (EC 3.4.23.4) and it cleaves the $Phenylalanine_{105}$-$Methionine_{106}$ bond of κ-casein (Delfour *et al.* 1965 according to Fox *et al.*, 1996). The cleaving of this bond in casein combined with the low pH from the lactic acid, causes the colloidal suspension of casein to collapse to form a coagulum or gel (Fox, 1981). A low pH is also needed for the proteolytic action of chymosin to be highly effective with the optimum activity of chymosin near pH 4 (Fox *et al.*, 1996). The coagulum is then cut, heated and the whey drained off and the resultant gel is called curd. For Cheddar cheese there is an extra process called Cheddaring that involves stacking the cut blocks of curd to express more of the whey as well as to complete lactic acid production. After Cheddaring the curd is milled and salt added before pressing into moulds and then storing for ripening (Scott, 1986). Fox *et al.* (1995a) mentioned that 6% of the rennet is retained in the curd after the curd is pressed and this plays a major role in the initial proteolysis of caseins in many cheese varieties yielding mostly macropeptides (O'Keeffe, Fox & Daly, 1978)

Chymosin is rather weakly proteolytic (Fox, 1989) yet it cleaves the $Phenylalanine_{105}$-$Methionine_{106}$ bond of κ-casein because it has been found to be many times more susceptible to hydrolysis than any other casein peptide bond (Fox *et al.*, 1996). Why this bond in particular is so susceptible to hydrolysis is not well understood (Fox, 1981) though it appears as if the amino acid sequence surrounding the bond causes its susceptibility (Fox *et al.*, 1996). The primary task of chymosin is to clot the milk yet many other proteases that clot milk cause a higher degree of proteolysis relative to their clotting ability, leading to reduced cheese yields and/or inferior quality cheese (Fox *et al.*, 1996). The secondary task of chymosin is to continue proteolysis during ripening which contributes to the flavour of the cheese.

Rennet is an animal product derived from the stomachs of suckling calves. Increased demand for cheese as well as a lowering of the cull rate of calves has lead to a shortage of rennet (Fox, 1981). Rennet from other sources has been investigated with the following rennets being found suitable: bovine, porcine and chicken pepsins and the acid proteases of *Rhizomucor miehei*, *R. pusillus* and *Cryphonectria parasitica* (Fox *et al.*, 1996). Companies like Gist Brocades, Pfizer and Hansen's have also cloned the gene for calf chymosin into microorganisms and the chymosin produced from these microorganisms is steadily gaining acceptance in several countries (Fox *et al.*, 1996). Historically, non-animal enzymes (including those from cloned bacteria) have not been as effective as animal enzymes such as rennet and pre-gastric esterases in producing high quality cheese since their enzyme action is different (Coulson, Pawlett & Wivell, 1992).

Plasmin and cathepsin-D are proteases that naturally occur in milk. Plasmin (fibrinolysin, EC 3.4.21.7) is an alkaline enzyme and probably plays little part in the ripening of Cheddar cheese due to the low pH of cheese of about pH 5 (Fox *et al.*, 1996). Cathepsin-D has a similar specificity to that of chymosin yet it coagulates milk very slowly (McSweeney *et al.*, 1995 according to Fox *et al.*, 1996). Since cathepsin-D is a weak protease (as compared to chymosin) yet similar to chymosin, its effects can either be ignored or grouped together with that of chymosin. Another protease has been found by Kaminogawa & Yamauchi (1972) which is an acid protease similar to cathepsin-D with an optimum at pH 4 and with an enzyme action that is similar to that of chymosin.

The LAB are weakly proteolytic though they have the ability to break down the casein all the way to FAA (Moskowitz & La Belle, 1981; Law, 1984). Fox *et al.* (1996) stated that the starter LAB bacteria are considered crucial for cheese flavour development and according to Fox *et al.* (1995a), LAB are the major source of proteases and peptidases in cheese apart from chymosin. NSLAB and secondary starter microorganisms have a range of proteases and peptidases that are also involved in the proteolytic ripening of cheese (Fox *et al.*, 1996). The bacteria in the cheese lyse after death during the ripening of cheese, releasing aminopeptides, dipeptides and tripeptides which contribute to the level of FAA in the cheese (Law, Sharpe & Reiter, 1974; Fox & Wallace, 1997). Law, Andrews, Cliffe, Sharpe & Chapman (1979) in contrast found that no significant change in cheese quality, yield or

proteolysis could be detected if they added up to 10^7 cfu/ml of psychrotrophic non-starter bacteria to milk prior to cheesemaking.

The caseins in cheese curd are bland and do not add directly to cheese flavour since they are too high in molecular weight, but the peptides, FAA and their breakdown products do contribute to cheese flavour (Ney, 1981). Some peptides are bitter and much research has been done to reduce the bitterness caused by these. It has been shown that if a peptide has a value of Q>5862 J/mol and a molecular weight less than 6000 Da it will be perceived as being bitter, where Q is defined as the free energy of formation of the constituent amino acids with glycine being assigned a heat of formation of 0 J/mol (Ney, 1981; Fox *et al.*, 1996). Some bitterness is required in cheese since that is the nature of the product but excess bitterness will create an undesirable product. A number of authors (Aston & Creamer, 1986; Cliffe, Marks & Mulholland, 1993; Engels & Visser, 1994) have shown that peptides <500 Da make a significant contribution to Cheddar flavour and McGugan, Emmons & Larmond (1979) stated that peptides are also important since they can bind onto flavour compounds. Any excess bitterness can be eliminated either by reducing/controlling the degree of proteolysis or by the addition of peptidases. The bitterness rule mentioned above can also be applied to individual amino acids to show that some of them are bitter, as can be seen in Table 2 (Ney, 1981).

Approximately 3% of the total nitrogen content of cheese is present as FAA nitrogen (Law, Castañón & Sharpe, 1976). FAA on their own contribute directly to cheese flavour, though their contribution is probably small (Fox & Wallace, 1997). There has been disagreement over what the most important FAA in Cheddar flavour are: Marsili (1985) gives Glu, Met and Leu; Wood, Aston & Douglas (1985) give Leu, Glu, Asp, Lys and Phe; Engels & Visser (1994) give Glu, Leu and Phe and Aston & Creamer (1996) give Leu and Met.

FAA also play a part by serving as a substrate for the formation of flavour compounds (Fox & Wallace, 1997). Catabolism of FAA by microorganisms can result in a number of flavour compounds, as can be seen in Figure 2. Manning (1979) has shown that certain sulphur-containing compounds (methanethiol and hydrogen sulphide) could be produced in cheese with no starter, by lowering the redox potential of cheese, implying that they are produced chemically rather than by microbial

Table 2 Heat of formation of amino acids (Fox & Wallace, 1997 adapted from O'Callaghan, 1994)

Amino acid	*Q (J/mol)	Amino acid	*Q (J/mol)
Serine	-1256	Lysine	6280
Aspartic acid	0	Valine	6280
Glutamic acid	0	Leucine	7536
Glycine	0	Tyrosine	9630
Threonine	1675	Phenylalanine	10467
Alanine	2093	Proline	10886
Histidine	2093	Isoleucine	12351
Arginine	3140	Tryptophan	14235
Methione	5443		

*Q=free energy of formation with glycine defined as 0 J/mol

metabolism. Lowering the pH artificially without starter resulted in no flavour formation so Manning (1979) concluded that the low redox potential is required for flavour compound formation and/or preservation.

2.3.4 Lipolysis

Lipolysis in curd/cheese involves the breakdown of the milk lipids into diglycerides, monoglycerides and FFA. Some FFA are also synthesised by LAB, NSLAB and other bacteria from carbohydrate and protein in cheese but the bulk of evidence indicates that lipolysis is the principal contributor of FFA greater than C4 (Aston & Dulley, 1982). This is also borne out by the fact that skim milk cheese fails to develop cheese flavour (Harper, Kristoffersen & Wang, 1978). West & Pawlett (1996) differentiated between esterases and lipases by defining esterases as those enzymes which hydrolyse water-soluble esters and lipases as those enzymes which hydrolyse the ester bonds in oils and fats. For this discussion on lipolysis no distinction will be made between esterases and lipases.

Lipids can be degraded either by lipolysis or by oxidation but the possible contribution of oxidation to good cheese flavour has been largely ignored since it only occurs to a small degree (Fox & Wallace, 1997). Typical concentrations of total FFA are 1000 mg/kg for Cheddar and 30 000 mg/kg for Roquefort cheese (Fox & Wallace, 1997) which is well above the typical aroma and flavour threshold of 0.3 to 100 mg/kg for the range of fatty acids (Baldwin, Cloniger & Lindsay, 1973). Even though lipolysis may not be the major event in Cheddar cheese flavour (see section on proteolysis) it is still

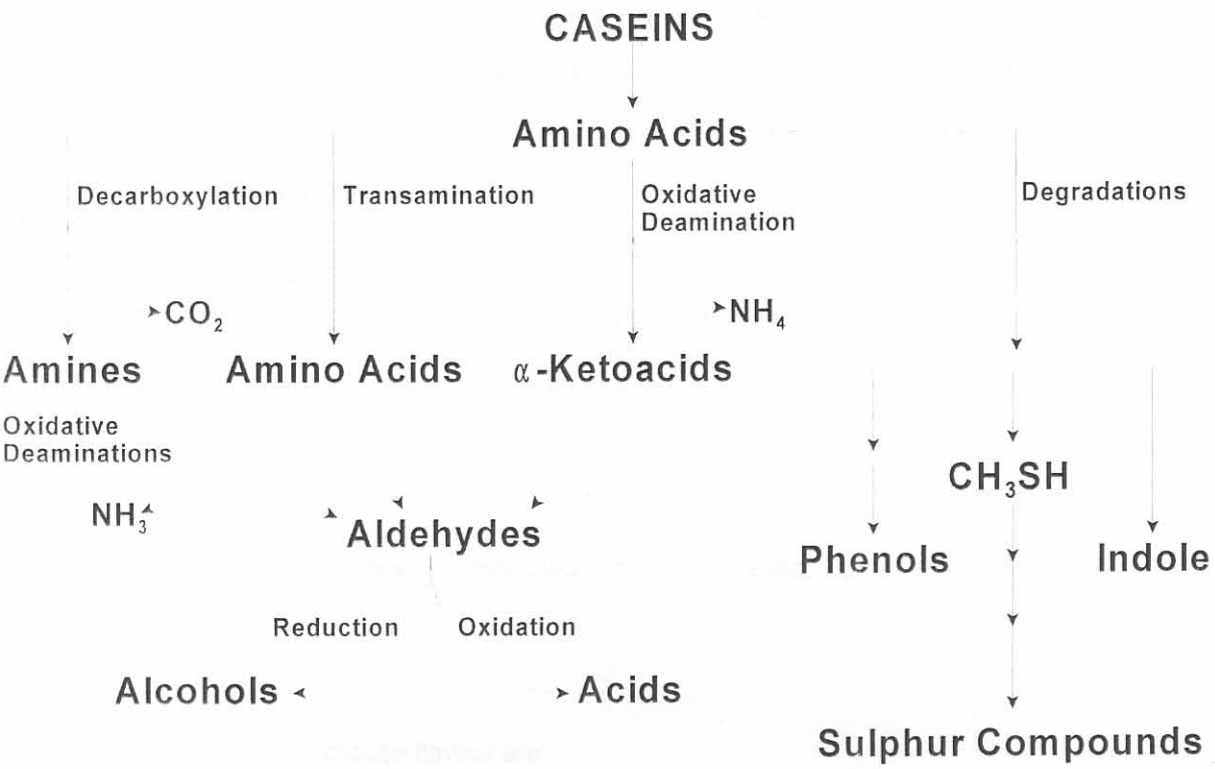

Figure 2 General pathways of microbial catabolism of amino acids during cheese ripening (Fox *et al.*, 1995a adapted from Hemme *et al.*, 1982)

important since cheese made with skim milk fails to develop flavour and low-fat Cheddar cheese has poor flavour (Ohren & Tuckey, 1969 according to Harper *et al.*, 1978).

Milk contains a well-characterised lipoprotein lipase (Fox & Wallace, 1997) which causes significant lipolysis in raw milk cheese but is mostly deactivated during pasteurisation. In Cheddar production the rennet is virtually lipase free (Fox & Wallace, 1997) and so the lipases must come from other sources. Lipases from psychrotrophic bacteria contribute significantly since they are heat stable and are concentrated in the curd during curd formation since they adsorb onto the fat globules (Foda, Hammond, Reinbold & Hotchkiss, 1974; Fox & Wallace, 1997).

Much work has been done on the importance of FFA to Cheddar cheese, there is not much agreement on which and how much FFA is important except that an excess causes rancidity. Kristoffersen (1967) and Singh & Kristoffersen (1970) stated that FFA are of key importance since FFA increase with flavour but Reiter *et al.* (1967) found no relationship between flavour and FFA. Milk fat is also important as a solvent for sapid compounds produced from other constituents (Fox &

Wallace, 1997). The FFA are broken down into ketones, lactones and other compounds, though these are of more importance in blue cheese flavour development (Fox & Wallace, 1997).

2.3.5 Other factors, including off-flavours

As mentioned previously, physical parameters such as pH affect the formation of cheese flavour. These physical parameters affect the microorganisms, added and indigenous enzymes as well as the inter-reactions between the products of the previous processes. These physical parameters include Eh, pH, water activity and salt content. Salt is also a taste compound in its own right but its action is relatively well known. Many other reactions involving the products from lipid, protein and carbohydrate metabolism/breakdown have been investigated (Fox *et al.*, 1995a; Fox *et al.*, 1996; Fox & Wallace, 1997).

Some common defects in cheese flavour are:

Bitterness - probably the principal defect in cheese, which is caused mainly by the accumulation of small peptides which are relatively rich in hydrophobic residues (Fox *et al.*, 1995a). FAA and their breakdown products can also contribute to bitterness (Fox *et al.*, 1996).

Astringency - a result of reactions between tannins and proteins in saliva (Lindsay, 1985).

Fruitiness - caused by reactions between ethanol and FFA to form esters (e.g. ethyl hexanoate and ethyl octanoate) (Ney, 1981; Fox & Wallace, 1997).

"Unclean" off-flavours - linked to Strecker-type products including phenylacetaldehyde, phenethanol, 3-methyl butanol, 2-methyl propanol, phenol and *p*-cresol (Dunn & Lindsay, 1985).

Rancidity - caused by excessive or unbalanced lipolysis.

Higher cleanliness in cheese manufacture today, as compared to traditional cheesemaking, has reduced the non-starter, citrate fermenting and other bacteria normally found in milk, with the result of a more consistent product but a milder flavoured cheese (Fox *et al.*, 1996). Work is being done to selectively add some of these bacteria in addition to the standard LAB starter but this is not yet common practice and is also legislated against in certain countries (International Dairy Federation, 1990). There is hope that with the selective addition of these indigenous bacteria a higher flavoured product will result, without any loss in quality.

2.4 ENZYME-MODIFIED CHEESE

As seen in the previous section the chemistry of cheese flavour formation is complex. If the multitude of known volatile Cheddar flavour profile chemicals (Table 3) are added to a bland base, the resultant product will not taste like Cheddar cheese due to as yet undetected, below threshold compounds (Nagodawithana, 1995). Even if the full reaction chemistry of Cheddar cheese flavour were known, the cost of producing it synthetically would probably be prohibitive (Kilcawley, Wilkinson & Fox, 1998). Therefore, enhancement of the major flavour pathways that occur in natural cheese presently provides the most viable route to the production of intense cheese flavours. This is best achieved by the enzymatic modification of cheese to produce EMC (Kilcawley *et al.*, 1998). Fox (1998) stated that the flavour from EMC does not approximate natural cheese flavour but rather has the ability to potentiate cheese flavour in various foods.

2.4.1 Enzyme modified Cheddar cheese chemistry

Proteolysis is the major event in Cheddar cheese flavour development (see section on Cheddar flavour development) but lipolysis is the major event in the production of Cheddar EMC (BioCatalysts, 1996). Godfrey & Hawkins (1991) reported that lipases can eliminate the need for the extensive protein hydrolysis, which is needed for strong flavour but which can also result in bitterness. For EMC production Kilara (1985) gave the activity ratios of protease to lipase as 1:2 to 1:3. As mentioned previously, EMC is a highly accelerated ripened cheese product and at present there are four ways that cheese can be ripened more quickly than normal (Table 4). As will be seen in the next section, EMC is produced using a combination of the first, second and fourth methods.

Kristoffersen *et al.* (1967) are quoted by many authors as the inventors of EMC through their investigations of the curd slurry technique (Sutherland, 1991; Thakar & Upadhyay, 1992; Kilcawley *et al.*, 1998). In their landmark study, curd was blended with saline solution (same salinity as in the curd, i.e. 5% NaCl in the moisture) and 100 mg/kg glutathione (GSH) and then stored at 30°C for 4 to 5 days with routine sampling. The resulting paste had the flavour of a mild Cheddar cheese and the authors commented that the product was ideally suited for use in processed foods. These techniques form the basis of EMC production today, though as the name implies enzymes are now also added.

Table 3 Volatile compounds important for Cheddar cheese flavour (Fox & Wallace, 1997)

Acetaldehyde	Dimethyl disulphide	2-Methylbutanol
Acetoin	Dimethyl trisulfide	3-Methybutanol
Acetone	δ-Dodecalactone	3-methyl-2-butanone
Acetophenone	Ethanol	3-Methylbutyric acid
β-Angelicalactone	Ethyl acetate	2-Nonanone
1,2-Butanediol	2-Ethyl butanol	δ-Octalactone
n-Butanol	Ethyl butyrate	n-Octanoic acid
2-Butanol	Ethyl hexanoate	2-Octanol
Butanone	2-Heptanone	2,4-Pentanediol
n-Butyl acetate	n-Hexanoic acid	2-Pentanol
n-Butyric acid	n-Hexanol	Pentan-2-one
Carbon Dioxide	2-Hexanone	n-Propanol
p-Cresol	Hexanethiol	Propanal
γ-Decalactone	2-Hexenal	Propenal
δ-Decalactone	Isobutanol	n-Propyl butyrate
n-Decanoic acid	Isohexanal	Tetrahydrofuran
Diacetyl	Methanethiol	Thiophene-2-aldehyde
Diethyl ether	Methional	2-Tridecanone
Dimethyl sulphide	Methyl acetate	2-Undecanone

Table 4 Major categories of methods for accelerating cheese ripening (adapted from Law & Goodenough, 1995; Fox *et al.*, 1996)

Method	Advantages	Disadvantages
1. Elevated temperature	No legal barriers, technical simplicity	Non-specific action, increased microbial spoilage potential
2. Enzyme addition	Low cost, specific action, choice of flavour options	Limited source of useful enzymes, danger of over-ripening, difficult to incorporate
3. Modified starter	Probably no legal barriers, natural enzyme balance retained, easy to incorporate	Technical complexity, uneconomical at present
4. Cheese/curd slurries	Very rapid flavour development, used to study new enzymes	Cannot be used to accelerate flavour in solid cheese

EMCs are produced using curd slurries since this is the quickest procedure and texture is not important (Fox *et al.*, 1996). Thakar & Upadhyay (1992) have reviewed the curd slurry technique and conclude that the major biochemical pathways of flavour formation in curd slurries and cheese are

similar. Curd slurries have been used as a method of testing new cheesemaking techniques and additives and EMC evolved from this model system (Kilcawley *et al.*, 1998).

EMC is made from cheese, cheese scraps or fresh curd (West & Pawlett, 1996). Some authors have added additional starter culture during the production process (Jang & Lee, 1985; Takafuji, 1993) but others have not and still produced acceptable product (West & Pawlett, 1996). Many authors have tried different additives in curd slurries to enhance flavour e.g. Singh & Kristoffersen (1970) added $CoCl_2$, sodium citrate, $MnSO_4$ and riboflavin and noticed a slight improvement in flavour. Singh & Kristoffersen (1971a) and Singh & Kristoffersen (1971b) reported that if the curd was made using direct acidification then starter culture, GSH, cobalt solution, riboflavin and diacetyl were needed as well as daily pH adjustment for development of Cheddar-like flavour in 8 days.

The one additive in EMC/curd slurries to enhance flavour that is reported more than any other is GSH (Kristoffersen *et al.*, 1967; Harper & Kristoffersen, 1970; Singh & Kristoffersen, 1971a; Manning, 1979; Kilara, 1985; Thakar & Upadhyay, 1992). Moskowitz & Noelck (1987) stated that GSH is important for flavour development by dissociating proteins, protecting esterases from degradation and maintaining flavour compounds in a reduced state. They also stated that GSH does not alter the redox potential but rather enhances production of H_2S and methanethiol and thereby enhances cheese flavour. Cysteine can be used as a substitute for GSH with comparable results (Samples, Dill, Richter & Dill, 1986).

Buhler (1995) in a trade publication, stated that Cheese Buds (similar to Cheesebase in Table 1) is better than EMC since EMC leaves a bitter aftertaste. Vafiadis (1996) and West & Pawlett (1996) confirmed that EMC originally had problems with bitterness. This has been solved by better understanding and control of proteolysis and the addition of peptidases from *Bacillus subtilis* and *Aspergillus oryzae* or preparations containing enzymes similar to these such as Debitrase® (West & Pawlett, 1996).

2.4.2 EMC technology

The production of EMC remains as much an art as a science (Moskowitz & Noelck, 1987). A number of different methods can be used to produce EMC, depending on the EMC type, manufacturer's preference, product application and appearance of the end product (Kilcawley *et al.*, 1998). A major variation over the curd slurry technique invented by Kristoffersen *et al.* (1967) (apart from the addition of enzymes) is the use of cheese instead of curd as a starting substrate by some authors. A more authentic flavour is produced with curd though EMC can be made from waste/defective cheese scraps since texture is not important (West & Pawlett, 1996).

Takafuji (1993) detailed the design and investigations required to commercially produce a Gouda EMC. Natural, mature Gouda is shredded and then mixed with additives (water, LAB, salt, protease from *Penicillium camemberti* and kid/lamb pregastric esterase) before going into a holding tank. The paste is then stirred with a recirculating pump for 10 days before pasteurisation at 80°C to deactivate the added enzymes. The product is then homogenised and packaged into plastic bags.

Bush Boake Allen (according to Vafiadis, 1996) manufacture EMC using fresh curd direct from their sister company on the same site, add enzymes and then incubate for a set period of time. Heat is used to deactivate the enzymes with care being taken not to destroy the cheese flavour during the heating process. The time and temperature relationships during flavour production and pasteurisation are critical for a good quality product. In a trade article, Freund (1995) gave the temperature and incubation parameters for Bush Boake Allen's process as 30°C or above for a minimum of 24 hours.

Sutherland (1975) made curd slurries by macerating unpressed salted curd with added water and salt to give a finely ground slurry with 40% total solids and 3.2% salt. The curd slurry was then incubated at 25°C after addition of rennet, GSH and/or lipase. Sutherland (1991) commented that the dairy industry uses techniques similar to the curd slurry technique to produce highly flavoured cheese slurries.

West & Pawlett (1996) gave two methods of producing EMC based cheese flavourings, namely the component approach and the one-step process. The component approach involves the production of

several different components which are then mixed into a final product. As an example, hydrolysed cheese curd (protein derived component) could be mixed with lipolysed cream (lipid derived component) to get an EMC. The component approach has a better control of product through variation of the blend. The one step process has an advantage in that certain flavour forming reactions occur synergistically (see section on Cheddar flavour formation for more details) and a more authentic flavour can be produced (Fox & Stepaniak, 1993). West & Pawlett (1996) demonstrated this synergism by showing the increase in lipolytic action of a lipase when used in conjunction with a protease (Savorase®) as compared to the lipase on its own. They gave a method of EMC production as:

1. Make a slurry (40-55% dry solids) using curd or cheese off-cuts
2. Heat treatment at 72°C for 10 min
3. Cool to 40-55°C and add lipase and/or protease
4. Incubate at 40-55°C for 8-36 h
5. Heat treatment at 72°C for 25-35 min

As can be seen from the method, EMC development now can take between a few hours and a few days (Vafiadis, 1996) while traditional production of cheese flavour used to require several months or more (Kilara, 1985).

Takafuji (1993) went through the long process of extracting, selecting and determining the correct concentration of enzymes to be used for EMC production. This takes a long time and though large companies may have the resources for such investigations, small companies do not. Many companies including Amano Enzymes, BioCatalysts, Imperial Biotechnology, Novo Nordisk and Röhm market purified enzyme preparations extracted from organisms such as *Aspergillus niger*, the recipes of which are not in the public domain (Kilara, 1985; Sutherland, 1991).

BioCatalysts produce a range of enzyme preparations for EMC production (BioCatalysts, 1996). The method of EMC production given by West & Pawlett (1996) is identical to that given by BioCatalysts since West works for BioCatalysts. The most commonly used enzymes from BioCatalysts for EMC production are Lipomod 187 (lipase) and Promod 215 (protease), both which are of microbial origin

and as such have Kosher and vegetarian status (pers. comm. - S. West, Director of BioCatalysts, Pontypridd, Wales, 1998).

2.4.3 Factors affecting EMC quality

For a high quality product, Talbott & McCord (1981) stated that temperature, pH and agitation must be tightly controlled and the progress of the reactions monitored such that the reaction is stopped when the product is at optimum quality. The time and temperature control during product pasteurisation is crucial to preserve the flavour while removing residual enzyme activity which would spoil any product that the EMC was used in (Vafiadis, 1996).

As mentioned previously, fresh salted curd must be used for the most authentic flavour and GSH addition is recommended for good Cheddar flavour. Since there will be many peptides produced in the curd-slurry, bitterness may be a problem but this can be eliminated by the addition of debittering enzymes e.g. Debitrase® (West & Pawlett, 1996). Horwood, Shanley & Sutherland (1994) mentioned that headspace oxygen increases the chance of fruity off-flavours in curd slurries, implying that either vacuum packaging or a nitrogen blanket must be used for EMC production. Spoilage by bacteria and yeast is a major problem due to the relatively high processing temperatures. Potassium sorbate (Dulley, 1976) and nitrates, sorbic acid and nisin (Mann, 1993) can be used to reduce microbial growth depending on legislation governing their use.

Kilcawley *et al.* (1998) published a comprehensive review on EMC. In their review they gave the key factors involved in EMC production as: the type of cheese required which is directly related to the starting material, type and specificity of enzymes or cultures used, their concentration, processing parameters (pH, temperature, agitation, aeration and incubation time) and use of processing aids (emulsifiers, bacteriocins, flavour compounds and precursors). The dosage of enzyme and/or starter culture used is dependent on the intensity of flavour required, processing time and temperature and the quality of the substrate. Kilara (1985) gave a table of factors that affect the enzymatic hydrolysis of proteins (Table 5). Most of these factors can also applied to lipases since they are parameters that pertain to enzymes in general.

Table 5 Factors affecting the enzymatic hydrolysis of proteins (Kilara, 1985)

Factors	Comments
Enzyme specificity	No single protease completely hydrolyses a protein. Mixtures of proteases are used.
Extent of protein denaturation	Denatured proteins are more susceptible to hydrolysis.
Substrate and enzyme concentration	Should be controlled.
pH	Optimum varies with enzyme.
Temperature	Preferably >45°C.
Ionic strength	Critical but neglected parameter.
Inhibitory substance	Should be absent.

2.5 NON-SENSORY METHODS FOR DETERMINING CHEESE FLAVOUR QUALITY

As previously discussed in the section on Cheddar cheese flavour, there is no chemical or class of chemicals that can be considered as the basis or fundamental for Cheddar cheese flavour. Fox & Wallace (1997) gave a list of volatile compounds that are known to contribute to Cheddar cheese flavour (Table 3) though if these chemicals were all added together they would not equate with good cheese flavour (Nagodawithana, 1995). This implies that any test for these chemicals or these classes of chemicals will be unable to determine whether a cheese is of high or low quality (Manning, Ridout & Price, 1984). This must be done subjectively by taste panels (Fox & Wallace, 1997). The ideal instrument would be a "cheese quality meter" but this is not available at present. A more realistic goal would be to produce low-cost and simple methods for the determination of the key parameter(s) in cheese that would be helpful as a guide in the evaluating cheese quality.

The development of gas chromatography in the 1950s and its interfacing with mass spectrometry has given scientists the tools to track the chemical changes in cheese by measuring the volatiles given off by cheese (Fox & Wallace, 1997). The non-volatile components like peptides can be analysed by Reverse Phase - High Performance Liquid Chromatography (RP-HPLC). Most of these techniques are far too time consuming for routine analysis and much work has been done to find the key parameter(s) that will give an indication of the quality of the cheese being tested. Manning *et al.* (1984) reviewed non-sensory methods for predicting cheese flavour quality including methods such as compositional analysis (salt, moisture and fat), volatile composition, redox potential and statistical

methodology. Few of the methods they mentioned are used commercially for grading cheese except compositional analysis, which has been successfully used in New Zealand since 1977.

Since the primary events in cheese and EMC flavour formation are proteolysis and lipolysis, measuring the degree of both of these will give some degree of understanding as to what is occurring during flavour formation.

2.5.1 Proteolysis

Proteolysis is the breakdown of proteins to peptides and FAA and methods for determining these compounds should be able to estimate the degree of proteolysis. Fox, McSweeney & Singh (1995b) described non-specific methods that involve measuring the fraction of protein soluble in solvents such as urea solution, water, acid, $CaCl_2$ solution, NaCl solution, methanol, ethanol, trichloroacetic acid, ethanol, phosphotungstic acid (PTA) and others. The soluble fraction will contain varying amounts of peptides and FAA depending on the method e.g. PTA precipitates all peptides over 600 Da (Jarrett, Aston & Dulley, 1982). The resulting liquor after centrifuging is then analysed for nitrogen content using the Kjeldahl or other method. Fox *et al.* (1995b) described direct methods for proteolysis determination including the measurement of ammoniacal nitrogen (using Nesslerization), soluble tyrosine/tryptophan, buffering capacity (which increases with proteolysis) and spectrophotometric determination of dyes which bond selectively to amino acids and/or amines e.g. ninhydrin. They also stated that commercial amino acid analysers based on ion-exchange chromatography combined with ninhydrin are used to quantify individual amino acids concentrations.

Techniques that can separate individual peptides are available such as electrophoresis or chromatography. Chromatography has been done in varying forms including paper, silica gel, metal chelate, Sephadex gel, ion exchange and RP-HPLC though HPLC techniques are still confined to the research laboratory (Fox *et al.*, 1995b).

2.5.2 Lipolysis

Deeth, Fitzgerald & Snow (1983) described a GC technique for the quantitative determination of FFA. A hexane-diethyl ether extract of cheese is prepared in the presence of anhydrous sodium sulphate

and passed through a column of neutral, deactivated alumina to separate fat. The FFA retained on the alumina are eluted with a small volume of 6% formic acid in di-isopropyl ether which is then evaluated using a GC. Shanley *et al.* (1979) (according to Horwood *et al.*, 1994) gave a similar but more complex method. Other methods that have been used historically (e.g. conversion of FFA to methyl esters followed by evaluation with a GC) can cause hydrolysis of the lipids during sample preparation which can lead to erroneous results (Deeth *et al.*, 1983).

There is a standard method for measuring total FFA in fat, which involves the titration of the product in hot neutral ethyl alcohol (Skoog, West & Holler, 1988).

2.5.3 Off-flavours

Of the factors listed in the section on off-flavours in cheese (bitterness, astringency, fruitiness, unclean flavour and rancidity), fruitiness (esters from ethanol and FFA reactions) and rancidity (lipolysis) are relatively easy to determine. Ethanol production is the rate-limiting step in ester formation so measuring this will give a guide to fruitiness in the ripened cheese (Fox & Wallace, 1997). Lipolytic rancidity is caused by the FFA levels being too high and so this can be measured by the tests mentioned in the section on lipolysis.

Peptide determination for bitterness using a chromatographic method is a long process and is not suitable for routine analysis. Cliffe & Law (1990) used RP-HPLC to study enzyme-treated Cheddar cheese slurries and confirmed that bitter peptides are relatively rich in hydrophobic residues. The bitter peptides exhibited higher retention on the reverse phase column.

Astringency and unclean flavours are harder to detect since the chemistry of formation is not well known and therefore any analytical test would not be able to guarantee an absence of the defect.

2.6 SENSORY METHODS FOR DETERMINING CHEESE FLAVOUR QUALITY

Cheese used to be assessed for flavour at least twice between manufacture and marketing to determine the most suitable marketing stage for the cheese, with only the best quality cheeses being ripened for up to 12 months. In the modern dairy industry, mature cheese is specifically produced but

still must be taste tested before being released for sale. There are three types of taste testing, namely expert panels, consumer panels and descriptive analysis taste panels. Since cheese flavour is similar to EMC flavour the same methods that apply to cheese can be applied to EMC.

2.6.1 Expert panels

The least scientific of the three types of taste testing is the expert panel since normally it is done by three to five people and little (if any) statistical analysis is done on the results. Expert panels are used for the 'day to day' quality determination of cheese and are commonly called cheese graders. Grading for cheese is normally done under three classifications: flavour and aroma, body and texture, and colour. Manning *et al.* (1984) commented that by and large they do an effective job. McBride & Hall (1979) found no correlation between consumer preferences and expert grading though this is to be expected since the two methods used would yield different results. Non-sensory (analytical) methods are being investigated as ways to replace expert graders though at present this is not possible since as discussed in the section on Cheddar cheese flavour the exact compounds that lead to good flavour are not known.

2.6.2 Consumer panels

Consumer panels involve getting the public to taste samples of product to see whether they like the product, prefer it over another product or just find the product acceptable (Heymann, 1995a). Since this is not a descriptive technique the only result would be ranking of products or an indication of product acceptability while no key is given as to why the product is acceptable. Consumer panels are normally only done towards the end of product development and after descriptive analysis had been done (Heymann, 1995a).

2.6.3 Descriptive analysis taste panels

Descriptive analysis is the most scientific of the three methods mentioned. Techniques such as quantitative descriptive analysis (QDA), quantitative flavour profiling (QFP) and sensory spectrum are all descriptive analysis methods with various adaptations (Heymann, 1995b). Descriptive analysis involves describing the flavour using descriptors and there are two variations of the technique. In the first technique the panel generate the descriptors using reference standards and other products in the

category chosen. In the second technique the descriptors and references are provided. Heymann (1995b) used a combination of the two techniques where the panel generates the descriptors but the panel leader may modify the list by combining, adding or deleting terms based on prior experience with the product type. Stampanoni (1993) stated that the objective of descriptive analysis is to find a minimum number of descriptors that will convey a maximum amount of information regarding the sensory characteristics of the product. The use of commonly available flavour/aroma references will allow the comparison of different studies, which is not possible using either consumer or expert panels.

Samples for descriptive analysis are initially done in triplicate to determine judge repeatability and thereafter the product must be tested in duplicate but preferably in triplicate. All the standard sensory practices including sample coding, randomised serving sequences and use of individual booths should be employed during the judging (Heymann, 1995b). The use of non-numerical scales is recommended by Stone & Sidel (1985) (according to Stampanoni, 1993).

2.7 CONCLUSION

Cheddar cheese is used as an ingredient in many foodstuffs since it has a good savoury flavour. In Australia for example, 50% of cheese is either processed or used in factory produced foods. EMC has many times the flavour strength of cheese and is used by industry to reduce the cost of adding Cheddar cheese flavour to a product. The EMC is a slurry or paste and therefore has poor texture but this is unimportant in products such as sauces where only a good imparted flavour of Cheddar cheese is important. This means that the EMC does not necessarily need to have a balanced flavour in itself but rather needs to taste balanced once incorporated into a foodstuff.

The production of Cheddar cheese flavour is a complex process, starting with the simple breakdown of proteins, lipids and lactose by added enzymes such as chymosin and those enzymes from starter and non-starter bacteria and ending with inter-reactions of the reaction products and substrates. The keys to defining Cheddar cheese flavour appear to still be beyond our grasp. Therefore Cheddar cheese flavour still cannot be made artificially, directly from pure chemicals, and hence the need for an accelerated ripened cheese product such as EMC. The chemistry of off-flavour development is

easier to define since it is normally the result of a few compounds or a variation in an easily measured parameter such as pH.

The chemistry of Cheddar EMC flavour formation is similar to that of Cheddar cheese though lipolysis is reported to be more important during flavour formation than proteolysis as compared to Cheddar where proteolysis is dominant. The technology used for EMC production has evolved from the early work done by Kristoffersen (1967) which involved incubating curd slurries at elevated temperatures for a few days. The modifications to the curd slurry technique allow for the addition of enzymes, the option of using cheese instead of curd as the substrate and the addition of other compounds such as preservatives. To make matters more complex, most EMC production is carried out using commercial enzyme preparations of unknown recipes since the art of producing and extracting enzymes would probably not be feasible for an EMC producer. The technology of commercial EMC production is complex and as with cheese production the variations in recipes and technologies are numerous and are largely dependent on what product is required.

EMC is heat treated after production to make a shelf stable product by denaturing the added and endogenous enzymes. This heat treatment is vital to make a stable product but since EMC is composed of many flavour compounds of varying heat stability, heat damage must be limited by good control of the heating process.

Non-sensory based tests are unable to guarantee good quality Cheddar cheese due the complexity of Cheddar cheese flavour. Tests that analyse for the progress of proteolysis, lipolysis and glycolysis will give some understanding as to the expected flavour quality but non-sensory based tests appear to only be good for detecting gross imbalances in flavour and the presence of off-flavour compounds.

The ultimate tester of good quality Cheddar flavour is the consumer so sensory testing will give the best indication of flavour quality. During the research and development phase of a Cheddar cheese product, descriptive analysis is most suitable to find out where the strengths and weakness of a product are. Once a variety of test products have been made they can be tested again with descriptive analysis or with difference tests that are normally carried out on people not associated with

the production of the product. Finally, expert graders are used for the daily quality control and grading of flavour once the production phase has been reached.